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IdeS, a Highly Specific Immunoglobulin G (IgG)-Cleaving Enzyme from *Streptococcus pyogenes*, Is Inhibited by Specific IgG Antibodies Generated during Infection

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IdeS, a recently discovered cysteine proteinase secreted by the important human pathogen *Streptococcus pyogenes*, interferes with phagocytic killing by specifically cleaving the heavy chain of immunoglobulin G. The fact that the enzyme targets one of the key molecules of the adapted immune response raised the question of whether an antibody response against IdeS could inhibit, i.e., neutralize, enzyme activity. Paired acute- and convalescent-phase serum samples from patients with pharyngotonsillitis ($n = 10$), bacteremia ($n = 7$), and erysipelas ($n = 4$) were analyzed. Antibodies with the ability to neutralize IdeS enzymatic activity were already found in two-thirds of acute-phase sera. However, patients who seroconverted to IdeS, in particular patients with pharyngotonsillitis and erysipelas, developed specific antibodies during convalescence with an increased capability to efficiently neutralize the enzymatic activity of IdeS. Also, the presence of neutralizing antibodies decreased the ability of IdeS to mediate bacterial survival in human immune blood. In patients with bacteremia, several acute-phase sera contained neutralizing antibodies, but no correlation was found to severity or outcome of invasive infections. Still, the fact that the human immune response targets the enzymatic activity of IdeS supports the view that the enzyme plays an important role during streptococcal infection.

Streptococcus pyogenes is an important human bacterial pathogen that causes a variety of diseases, including pharyngotonsillitis, impetigo, scarlet fever, septicemia, necrotizing fasciitis (NF), and streptococcal toxic shock syndrome (STSS) (6, 7). The survival of *S. pyogenes* depends on its ability to avoid the various actions of the human immune system. Immunoglobulin G (IgG) plays a key role in the immune defense by specifically recognizing invading microorganisms and mediating their killing by professional phagocytes and the complement system. In order to persist, pathogenic bacteria have to find ways to avoid recognition by immunoglobulins and to interfere with IgFc-mediated phagocytosis. *S. pyogenes* has evolved a specific enzyme to deal with opsonizing IgG antibodies. This enzyme, designated IdeS or streptococcal Mac-1 (12, 23), is a secreted cysteine proteinase that specifically cleaves the heavy chain of IgG (1, 22, 23). So far, no other substrates for IdeS have been identified, and IgG is the sole substrate of IdeS in plasma samples (22). Due to its early and sustained expression during growth (23) and its highly specific proteolytic activity, IdeS is a tailor-made defense against Fc-mediated phagocytic killing (1, 12, 13, 22–25). Two protein variants of IdeS, complex I and complex II, have been described based on differences in the amino acid sequences of the middle thirds of the proteins (amino acids 112 to 205) (13). Complex II variants have been reported to have weak endopeptidase activity and to interfere with phagocytic killing by blocking the interaction of the FcγIIIb receptor with specific antibodies (1, 13), while complex I variants exert their inhibitory function through proteolytic cleavage of IgG (1).

The expression of streptococcal virulence factors in vivo is commonly analyzed by the determination of antibody levels towards particular streptococcal proteins in human blood samples. Paired acute- and convalescent-phase serum samples from children with streptococcal pharyngotonsillitis have been used to study the antibody response against the streptococcal C5a peptidase (17), and acute-phase serum IgG levels towards streptococcal M proteins and pyrogenic exotoxins (Spe's) have been analyzed and correlated to the outcome of disease (4, 10, 18). In another study, acute-phase serum samples of patients suffering from invasive streptococcal infections were analyzed for IgG antibodies towards six streptococcal virulence factors (SclA, SclB, MtsA, Grab, EndoS, and IdeS) (3). Detectable antibody levels against IdeS/Mac were found in this study as well as in two previous studies (12, 13). Other analyses of antistreptococcal antibodies, however, have shown that the total amount of specific antibodies does not necessarily correlate with the level of neutralizing antibodies, and that antibody quality might be clinically more important than antibody quantity (15, 16). The fact that *S. pyogenes* has evolved an enzyme that specifically targets IgG raises the question of whether specific IgG antibodies are able to neutralize IdeS and the further question of whether the presence of neutralizing antibodies correlates with manifestations of *S. pyogenes* infection or affects the severity of *S. pyogenes*-induced disease.

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TABLE 1. Characterization of *S. pyogenes* strains used in this study

Strain	Clinical diagnosis or source	M serotype[b]	IdeS complex
So1	Pharyngotonsillitis	77	I
So3	Pharyngotonsillitis	2	II
So4	Pharyngotonsillitis	Mnt	II
So5	Pharyngotonsillitis	12	II
So9	Pharyngotonsillitis	28	II
So14	Pharyngotonsillitis	1	I
So15	Pharyngotonsillitis	89	II
So16	Pharyngotonsillitis	2	II
So19	Pharyngotonsillitis	4	I
So22	Pharyngotonsillitis	28	II
Se1	Bacteremia	3	I
Se2	Bacteremia	122	II
Se3	Bacteremia	1	I
Se4	Bacteremia	8	II
Se5	Bacteremia	1	I
Se6	Bacteremia	78	II
Se7	Bacteremia	89	II
AP1	WHO Prague collection[a]	1	I

[a] WHO Collaborating Center for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic.
[b] Mnt, M nontypeable.

MATERIALS AND METHODS

Patients, serum samples, and bacterial isolates. Serum samples were collected from seven patients treated for *S. pyogenes* bacteremia at the Clinic for Infectious Diseases, Lund University Hospital, Lund, Sweden. Five of the patients had a nonsevere bacteremia, one patient developed necrotizing fasciitis, and one patient presented with STSS (26). *S. pyogenes* strains were isolated from blood cultures, and acute-phase serum (days 1 to 4 after onset of symptoms; median, day 1) and convalescent-phase serum (days 18 to 188 after onset; median, day 24) samples were collected from each patient. Sera from 10 patients with pharyngotonsillitis were collected at the Community Health Center Sorgenfrimottagningen, Malmö, Sweden. Acute-phase serum samples (taken between days 0 and 4 after onset of symptoms; median, day 1.8) and convalescent-phase serum samples (taken between days 24 and 30 after onset; median, day 26) were collected from each patient. *S. pyogenes* strains were isolated by throat swab cultures from all patients. Four patients treated for erysipelas at the Clinic for Infectious Diseases, Lund, Sweden, were also included in the study. They had typical signs of a bacterial skin infection, with fever and a rapid spreading of a painful erythema on a lower limb. From these patients, acute-phase sera were collected between days 0 and 5 after onset of symptoms (median, day 2.6), and convalescent-phase serum samples were taken between days 28 and 37 after onset (median, day 31). No bacterial isolate was available from the four erysipelas patients. Acute-phase serum samples from patients with either severe ($n = 4$) or nonsevere ($n = 4$) group A streptococcal invasive disease, and with high antibody titers towards IdeS, were from The Netherlands (1994 to 1997) and have been described elsewhere (3, 14).

Immunoglobulins. Human polyclonal IgG and myeloma IgG1(κ) were purchased from Sigma. IgG from patient serum was purified by affinity chromatography using HiTrap protein G Sepharose (Amersham Biosciences) according to the manufacturer's instructions. Myeloma IgG1 was labeled with ^{125}I using the Bolton and Hunter reagent as described by the manufacturer (Amersham Bioscience). Free ^{125}I was separated from labeled protein on a PD10 column (Amersham Bioscience).

Bacterial strains and growth conditions. *S. pyogenes* strains used in this study are listed in Table 1. Streptococci were routinely grown in Todd-Hewitt broth (TH) (Difco) or TH broth supplemented with 2 to 5% heparinized human plasma at 37°C in 5% CO_2. The M serotype of streptococcal strains was determined by sequencing *emm* gene-specific PCR products as previously described (5). Sequences obtained from *emm*-specific PCR products were subjected to homology searches in the *emm* sequence database at the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/emmtypes.htm; last accession, 20 February 2005). The sequence of bases ranging approximately from 65 to 165 obtained with primer 1, TATT(C/G)GCTTAGAAAAT TAA, has been implicated mostly as M type specific (5). Sequences that show greater than 95% identity with a reference *emm* gene sequence were considered to be given *emm* genes. Allelic forms of IdeS (13) were amplified as previously described (23), and sequences were determined by sequence analysis of nucleotides 121 to 900 of the *ideS* gene by use of an Applied Biosystems 3100 automated sequencer (Applied Biosystems). Multiple sequence alignment of the internal amino acid sequence of IdeS was conducted with CLUSTAL W (20).

Expression and purification of recombinant IdeS. The expression of recombinant IdeS in *Escherichia coli* has been described previously (23). Fusion proteins were purified on glutathione-Sepharose (Amersham Biosciences) according to standard protocols. IdeS protein of the complex II family was subcloned from the M8 serotype strain Se4 and purified as described above (for IdeS variants used in this study, see Fig. 2C). IdeS-304 and IdeS-Δ218 were created by PCR with primers I (23) and III (5′-TTCTTTGAATTCTTAAGCAGAAATAGCTA CTTTTCC-3′) for IdeS-304 and with primers IV (5′-GGTGATCAAAGGATC CTATTGACAAGTC-3′) and V (5′-CGGAATTCTTAATTGGTCTGATTCC AAC-3′) for IdeS-Δ218 by using IdeS inserted into a pGEX-5X-3 vector as the template (23). PCR fragments were cloned into the corresponding sites of plasmid pGEX-5X-3 (Amersham Biosciences). IdeS-232 and IdeS-Δ47-168 were created by digesting full-length *ideS* in pGEX5-3 with restriction endonuclease AvaI or StyI (Roche). The digested plasmids were purified, religated, and transformed into *E. coli* strain NovaBlue (Novagen). All variants were purified as glutathione *S*-transferase (GST) fusion proteins as described previously (23).

ELISA. Microtiter plates (Maxisorb; NUNC) were coated with enzymatically inactive IdeS^{C94G} (24) at 0.4 μg/ml in coating buffer (0.05 M $NaHCO_3$, pH 9.6). Plates were washed with PBST (0.05% Tween in phosphate-buffered saline [PBS]) and blocked with 2% bovine serum albumin (Sigma) in PBST (PBSTA). Dilutions of each patient and control serum sample in PBSTA were added to the wells. Antigen concentrations and serum dilutions were judged as optimal by checkerboard titration. Bound antibodies were detected by incubation with peroxidase-conjugated antibody against human IgG (1/3000) (Bio-Rad), and enzyme-linked immunosorbent assay (ELISA) plates were developed as previously described (3). Blank samples without serum were included in quadruplicate on each plate. Optical density at 420 nm (OD_{420}) was determined as described previously. An ELISA index was calculated by subtracting OD_{420} values of blank control samples from the values obtained with serum samples and by dividing the mean OD_{420} value for each sample by the mean OD_{420} values for a standard positive serum sample; these were determined in quadruplicate on each plate.

IdeS activity assays. For standard IdeS activity assays in bacterial growth medium, bacteria were grown to an OD_{620} of ~0.4 in TH broth supplemented with 2% heparinized human plasma and 40 μM of the cysteine proteinase inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64; Sigma) to block potential SpeB activity. Cleavage of IgG was determined by analyzing supernatant samples on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and IdeS activity was determined by the presence or absence of a diagnostic 31-kDa IgG cleavage product (1, 22, 23).

Quantification of IdeS activity. Quantification of the endopeptidase activity of IdeS was done as previously described for microbial IgA proteinases (9). Briefly, various amounts of the enzyme were incubated with ~10^5 cpm of ^{125}I-IgG and 4 μg of unlabeled myeloma IgG1 in PBS. Samples were kept at 37°C for 60 min, and the reactions were stopped by the addition of SDS sample buffer and incubation at 95°C for 5 min. IgG chains and IgG cleavage products were separated on 12% SDS-PAGE gels and visualized by staining with Coomassie blue (R-250; U.S. Biochemicals). Protein bands corresponding to the IgG heavy chain, IgG light chain, and the IdeS cleavage product (CP) were excised from the gel for determination of radioactivity. Samples were counted in an LKB Wallac Compugamma counter, and IdeS activity was calculated as the ratio of radioactivity in CP to total heavy chain radioactivity (CP plus heavy chain).

Detection of neutralizing antibodies in patient serum samples. Generally, IdeS was incubated with 5 to 10 μl of acute-phase or convalescent-phase serum samples at 25°C for 10 min. The reaction volume was adjusted to 28 μl with PBS, and ~10^5 cpm of ^{125}I-IgG was added as the substrate. Incubation was continued at 37°C for 45 min. The reactions were stopped by adding SDS sample buffer, and samples were analyzed by 12% SDS-PAGE. IdeS activity was determined as described above and correlated to enzyme activity in serum depleted of IgG. The IdeS endopeptidase activity was 27.5% cleavage of the IgG heavy chain under these experimental conditions, and this value was arbitrarily set as 100%. Activity in patient serum samples is expressed relative to this standard value.

Bactericidal assay. Purified IdeS (5 μg) was incubated with a three- to fivefold molar excess of either human polyclonal IgG (Sigma) or total patient IgG at 25°C for 30 min. As a control, IdeS^{C94S} (25) was incubated with a similar amount of human polyclonal IgG (Sigma). The suspension was added to 1 ml of heparinized human blood containing antibodies against *S. pyogenes*, and incubation was

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So3  212-FTRGDQSKLLTSRHDFKEKTLKEISDLIKKD----KALGLSHTYANVRINHVINLWGADFDSNGNL-277
So4  212-FTRGDQSKLLTSRHDFKEKTLKEISDLIKKD----KALGLSHTYANVRINHVINLWGADFDSNGNL-277
CII  212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTCANVRINHVINLWGADFDSNGNL-277
So9  212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTCANVRINHVINLWGADFDSNGNL-277
So22 212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTCANVRINHVINLWGADFDSNGNL-277
So5  212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYANVRINHVINLWGADFDSNGNL-277
Se4  212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYANVRINHVINLWGADFDSYGNL-277
So16 212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYANVRINHVINLWGADFDSNGNL-277
Se6  212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYAYVRINHVINLWGADFDSYGNL-277
Se7  212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYAYVRINHVINLWGADFDSYGNL-277
So15 212-FTRGDQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYAYVRINHVINLWGADFDSYGNL-277

                                    Y/C N/Y          N/Y
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FIG. 1. Alignment of complex II IdeS proteins analyzed in this study. The previously published sequence of complex II IdeS is designated CII (8). Amino acids 212 to 277 are shown. Sequence differences are boldfaced and variations indicated below the alignment. The additional cysteine residues in M28 serotypes are boxed.

continued for 60 min at 37°C with rotation. Approximately 500 CFU of a mid-log-phase culture of strain AP1 (OD_{620} of ~0.15) was used to inoculate the pretreated blood. The tubes were incubated with rotation at 37°C for 60 min, and bacterial survival was monitored by plating dilutions on blood agar plates.

RESULTS

Characterization of bacterial isolates. The M serotypes of the 17 clinical *S. pyogenes* isolates were determined by *emm* sequence analysis (5) and are listed in Table 1. Eight of the 11 different serotypes identified are among the most frequently identified M serotypes from clinical isolates (21). PCR analysis demonstrated the presence of the *ideS* gene in all isolates (data not shown). Strains of the same M serotype generally carried the two previously described (13) *ideS* alleles. Sequence comparison of the complex II IdeS variants found in the present clinical strains confirmed the reported high degree of sequence homology between these variants (13), with only a few amino acid substitutions. Two changes, however, appear to be of particular interest. (i) Strains So3 and So4 express an IdeS protein that is characterized by a four-amino-acid deletion in positions 243 to 246, distinguishing these proteins from all other IdeS variants. (ii) IdeS of the M28 serotype carries a cysteine residue in position 255. All other proteins, including complex I variants, carry a tyrosine residue at this position (Fig. 1).

IdeS antibody levels in acute-phase and convalescent-phase patient serum samples. IdeS-specific antibodies were detectable in serum samples of all patients (Table 2). Seroconversion was defined as an increase in the ELISA index between acute- and convalescent-phase sera of more than 40%. This was found in 10 of the 21 patients investigated, and in 5 of these the increase exceeded 100% (Table 2). For two patients, PS1 and PS4, high antibody levels towards IdeS had already been detected in acute-phase serum samples.

Specific IgG antibodies towards IdeS inhibit enzymatic activity. In a patient (PS2) who seroconverted to IdeS, no IdeS-mediated cleavage of IgG could be detected in convalescent-phase serum samples containing high levels of anti-IdeS antibodies (Fig. 2A; >90% inhibition of enzyme activity towards myeloma IgG1 in sample PS2C). However, when patient IgG was depleted from the sample and replaced by nonspecific polyclonal IgG, the myeloma IgG1 was efficiently cleaved, and no IdeS inhibitory activity could be detected (Fig. 2A, bar 3; 90% cleavage of myeloma IgG1). The serum regained its neutralizing ability when patient IgG was added back to the sample (Fig. 2A, bar 4; >78% inhibition). Thus, IgG antibodies towards IdeS clearly mediate the neutralization of enzymatic activity in serum samples.

In a second experiment, neutralizing convalescent-phase serum was incubated with an excess of inactive $IdeS^{C94S}$ (25) prior to the addition of active IdeS. The absorption of specific IdeS antibodies to inactive $IdeS^{C94S}$ efficiently abolished the neutralizing activity of convalescent-phase serum samples (Fig.

TABLE 2. Mean ELISA indexes towards IdeS in acute-phase and convalescent-phase serum samples

Patient[a]	Infecting strain	ELISA index for serum sample from:		Seroconversion[b]
		Acute phase	Convalescent phase	
PT1	So1	1.01	1.53	+
PT3	So3	0.45	0.53	−
PT4	So4	0.58	0.42	−
PT5	So5	0.57	0.59	−
PT9	So9	0.53	1.67	++
PT14	So14	0.35	0.29	−
PT15	So15	0.48	1.76	++
PT16	So16	1.22	1.08	−
PT19	So19	1.26	1.34	−
PT22	So22	0.56	1.84	++
PS1	Se1	2.31	3.04	−, H
PS2	Se2	0.42	1.52	++
PS3	Se3	0.53	0.78	+
PS4	Se4	2.40	1.71	−, H
PS5	Se5	0.37	0.44	−
PS6	Se6	0.78	1.31	+
PS7	Se7	0.81	2.30	++
PE1	Unknown	1.08	2.13	+
PE2	Unknown	1.63	2.48	+
PE3	Unknown	1.10	1.21	−
PE4	Unknown	1.43	1.24	−

[a] PT, PS, and PE indicate pharyngotonsillitis, sepsis, and erysipelas, respectively.

[b] H, high acute-phase titer; −, <40% increase; +, >40% increase; ++, >100% increase.

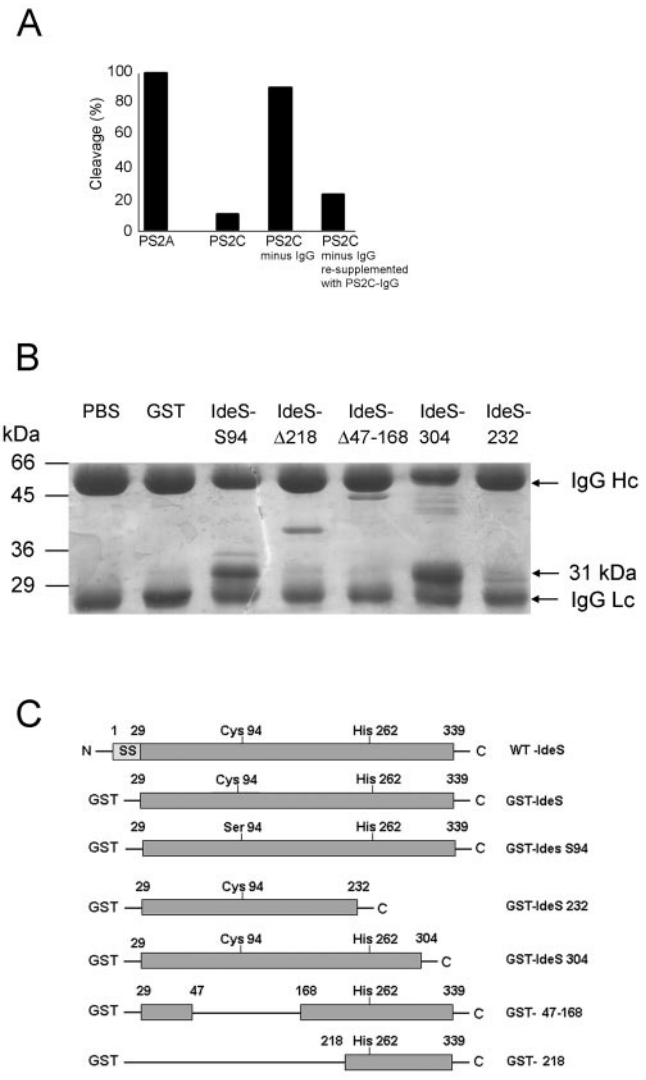

FIG. 2. Inactivation of IdeS enzymatic activity is mediated by antibody binding. (A) IdeS was added to the convalescent-phase serum sample from patient PS2 to determine neutralizing serum activity. ^{125}I-labeled myeloma IgG1 was used as the substrate, and enzyme activity was monitored by determination of the extent of ^{125}I-IgG1 cleavage. PS2A, acute-phase serum sample (bar 1); PS2C, convalescent-phase serum sample (bar 2). IgG was removed from PS2C, and the serum sample was resupplemented with either unspecific polyclonal IgG (bar 3) or the original convalescent-phase serum sample IgG (bar 4). (B) Convalescent-phase serum was preabsorbed with inactive IdeS variants prior to incubation with active enzyme. IdeS variants used are indicated above each lane. PBS (lane 1) and GST (lane 2) were used as controls. The position of the diagnostic 31-kDa cleavage product is indicated. (C) Graphic representation of IdeS variants used to absorb neutralizing antibodies.

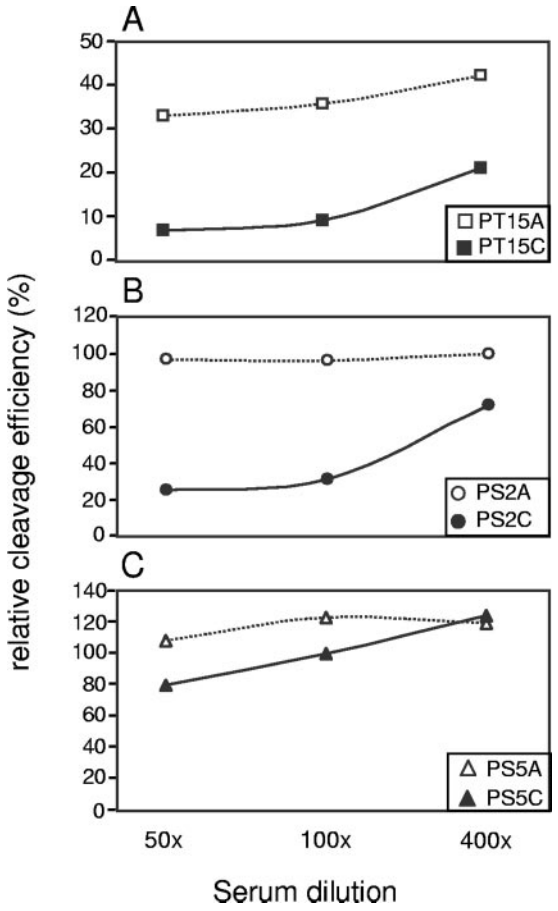

FIG. 3. Effect of convalescent-phase or acute-phase serum samples on IdeS activity towards myeloma IgG. Dilutions of patient serum samples were incubated with constant amounts of recombinant IdeS and ^{125}I-labeled myeloma IgG1 as the substrate. Sera from patients PT15 and PS2 had increases of >100% in ELISA index, and their patient PS5 showed a 19% increase in ELISA index. Inhibitions of IdeS activity in convalescent-phase serum samples at a 100-fold serum dilution were 83%, 83%, and 23%, respectively. Open symbols are acute-phase samples; filled symbols are convalescent-phase samples. Enzyme activity is relative to activity in serum depleted of IgG.

2B, lane 3; presence of 31-kDa cleavage product). Thus, the neutralizing activity in convalescent-phase serum samples is mediated by the binding of specific IgG antibodies to IdeS. Different enzymatically inactive truncation variants of IdeS (Fig. 2C and data not shown) were used to analyze the binding of neutralizing antibodies (Fig. 2B). Although the IdeS variants have retained the ability to bind IgG (data not shown; 11), only IdeS-304, containing both Cys94 and His262 active-site residues, absorbed neutralizing antibodies and mediated cleavage of IgG by active IdeS (Fig. 2B, GST-IdeS-304; presence of 31-kDa cleavage product). Thus, neutralizing activity in convalescent-phase serum samples is likely to be mediated by the binding of specific IgG antibodies to the catalytic region of IdeS.

Quality of patient antibodies. Studies of antistreptococcal antibodies have suggested that the quality of the antibodies, i.e., the neutralizing activity, may be clinically more relevant than the quantity of antibodies (15, 16). In an attempt to correlate the levels of antibodies towards IdeS with the degree of inactivation of IdeS, patient sera were incubated with IdeS, and the enzymatic activity was determined by measuring the cleavage of radiolabeled ^{125}I-labeled myeloma IgG1. This monoclonal IgG did not inhibit IdeS activity (data not shown). The results for two patients with a marked immune response (>100% increase in ELISA index; PT15 and PS2) and for one patient with only a moderate response (19% increase of

TABLE 3. Relative enzyme activity and degree of enzyme inhibition in acute- and convalescent-phase serum samples of patients diagnosed with pharyngotonsillitis, sepsis, or erysipelas[a]

Serum sample[b]	ELISA index ratio (C/A)	IdeS activity (%)	Inhibition (%)	Serum sample[b]	ELISA index ratio (C/A)	IdeS activity (%)	Inhibition (%)
PT1A	1.51	117	0	PS4A	0.71	26.9	73.1
PT1C		45.1	54.9	PS4C		44.4	55.6
PT3A	1.17	100	0	PS5A	1.19	120	0
PT3C		85.4	14.6	PS5C		92.7	7.3
PT4A	0.72	77.1	22.9	PS6A	1.68	30.2	69.8
PT4C		75.6	24.4	PS6C		34.2	65.8
PT5A	1.03	80.7	19.3	PS7A	2.84	68.7	31.3
PT5C		86.5	13.5	PS7C		82.5	17.5
PT9A	3.15	53.1	46.9	PE1A	1.97	116	0
PT9C		28	72	PE1C		25.8	74.2
PT14A	0.82	61.4	38.6	PE2A	1.52	36.4	63.6
PT14C		116	0	PE2C		17.8	82.2
PT15A	3.66	38.5	61.5	PE3A	1.10	134	0
PT15C		6.5	93.5	PE3C		122	0
PT16A	0.88	27.6	72.4	PE4A	0.87	88.4	11.6
PT16C		40.7	59.3	PE4C		109	0
PT19A	1.10	47.6	52.4	PS2A	3.62	100	0
PT19C		46.2	53.8	PS2C		17.4	82.6
PT22A	3.28	18.9	71.1	PS3A	1.47	52.7	47.3
PT22C		2.9	97.1	PS3C		96.7	3.3
PS1A	1.32	62.5	38.5				
PS1C		16.4	83.6				

[a] A, acute phase; C, convalescent phase. Inhibition of enzyme activity is relative to enzyme activity in serum lacking IgG. Values are averages for two independent experiments in duplicate at 100-fold serum dilution.
[b] PT, PS, and PE indicate pharyngotonsillitis, sepsis, and erysipelas, respectively.

ELISA index; PS5) are shown in Fig. 3A to C. Convalescent-phase serum samples from all three patients clearly inhibit IdeS activity (83%, 83%, and 23% inhibition compared to acute-phase serum at a 100-fold serum dilution). The inhibitory capacity of convalescent-phase serum samples diminished upon serum dilution, while IdeS activity remained unaffected in diluted acute-phase serum samples (Fig. 3A to C). Relative enzyme activity levels and the degrees of inhibition were determined for all patient serum samples (Table 3). In sera from patients with pharyngotonsillitis and erysipelas, an increase in the ELISA index correlated well with an increase in enzyme inhibition (Table 3). In contrast, seroconversion was not consistently reflected by an increase of enzyme inhibition when analyzing serum samples from bacteremia patients (Table 3; see data for PS3, PS6, and PS7). Furthermore, two-thirds of the analyzed sample pairs had already shown neutralizing activity in the acute-phase serum samples (Table 3). Still, serum samples from patients that did not seroconvert to IdeS showed no increase in neutralizing activity (Table 3), with the exception of the serum sample pair from patient PS1, which had already exhibited very high antibody titers in the acute-phase sample. It should be noted that the degree of inhibition, determined by analyzing the extent of ^{125}I-labeled myeloma IgG1 cleavage, is most accurate when comparing acute-phase and convalescent-phase serum samples from the same patient. Comparisons of inhibition levels between patients or patient groups are not practicable, as for instance 54.9% enzyme inhibition was sufficient to block patient IgG cleavage in serum sample PT1C, while 71.1% inhibition was not sufficient to block patient IgG cleavage in sample PT22A (data not shown).

Antibody quality and invasive streptococcal disease. Since acute-phase serum samples from sepsis patients notably differ in their abilities to neutralize IdeS activity, we speculated that the quality of preexisting antibodies could be important for the outcome of invasive streptococcal diseases. Acute-phase serum samples from patients with severe invasive infections ($n = 4$) and nonsevere invasive infections ($n = 4$), all with high levels of IdeS-specific antibodies (Table 4), were tested for their abilities to inactivate IdeS. Seven out of eight samples neutralized IdeS activity (data not shown), and there was no difference between the groups of samples from severe and nonsevere cases (Table 4). Thus, the presence of neutralizing antibodies towards IdeS does not appear to directly affect the severity of invasive streptococcal disease.

Neutralizing antibodies interfere with IdeS-mediated survival in human blood. IdeS mediates streptococcal survival in whole human immune blood by cleaving opsonizing IgG antibodies (23, 24). Although the presence of neutralizing antibodies towards IdeS does not affect the overall outcome of invasive streptococcal disease, such antibodies still could be detrimen-

TABLE 4. Neutralizing activity of acute-phase sera of patients with invasive *S. pyogenes* infections and high mean ELISA indexes towards IdeS

Patient	Disease	ELISA index	Neutralizing IgG
Ho1	Nonsevere invasive disease	3.46	+
Ho62	Nonsevere invasive disease	2.75	+
Ho67	Nonsevere invasive disease	2.95	+
Ho109	Nonsevere invasive disease	3.57	+
Ho32	STSS	2.87	+
Ho36	NF	2.31	−
Ho68	NF/STSS	2.37	+
Ho69	NF	3.00	+

tal for bacterial survival. The effect of neutralizing antibodies on streptococcal survival was assayed by using a human immune blood bactericidal assay. IdeS was incubated with either polyclonal or neutralizing IgG and added to immune blood prior to inoculation with *S. pyogenes* as previously described (23). Although the concentration of IdeS used in these experiments exceeded the amount of the enzyme used in the neutralizing experiments (25-fold), streptococcal survival was reduced by 58% in the presence of neutralizing antibodies (Fig. 4). Thus, neutralizing antibodies clearly affected the ability of IdeS to interfere with phagocytic killing of *S. pyogenes*.

DISCUSSION

S. pyogenes has to employ specific strategies to avoid the action of opsonizing IgG antibodies during infection. The streptococcal cysteine proteinase IdeS represents a tailor-made defense against specific IgG antibodies (1, 12, 13, 22–25) and is widely expressed by clinically important serotypes (e.g., M1, M3, M4, and M12), as well as in newly validated serotypes, e.g., M122 (2) suggesting an important role for IdeS during *S. pyogenes* infections.

The main focus of this study was to characterize the immune response towards the IgG endopeptidase IdeS in acute-phase and convalescent-phase serum samples from patients suffering from streptococcal disease. In accordance with previous data (3, 12, 13), detectable levels of antibodies against IdeS were found in all samples investigated. The neutralizing abilities of these antibodies varied notably between different patient samples, but consistent results were obtained for patients suffering from pharyngotonsillitis and erysipelas, revealing that patients who seroconverted to IdeS developed IgG antibodies with the ability to neutralize IdeS enzymatic activity. In contrast, patients with no or weak seroconversion have only poor neutralizing capability (Table 3). For patients treated for streptococcal sepsis, less consistent results were obtained, and seroconversion was not necessarily reflected by an increase of enzyme inhibitory activity (see results for PS3, PS6, and PS7). However, IdeS is expressed as one of two distinct protein variants (13), and neutralizing antibodies might be specific for one of the two IdeS variants. This fact might explain the lack of inhibitory activity in samples from patients PS6 and PS7 (see below). For two-thirds of the patients, neutralizing activities could already be detected in acute-phase serum. It appears unlikely that these specific antibodies are the result of a newly acquired IgG immune response this early after onset of infection (median, day 1). Instead, the ability of acute-phase serum to inactivate IdeS most likely reflects a preexisting immune response caused by previous *S. pyogenes* infections or colonization. This view is consistent with the fact that anti-IdeS antibodies are widely distributed in the healthy population (3).

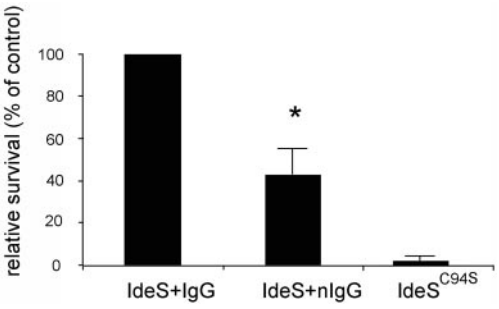

FIG. 4. Neutralizing antibodies interfere with IdeS-mediated survival in human blood. Whole human immune blood was treated with IdeS preincubated with polyclonal IgG (IgG) or purified neutralizing IgG (nIgG). *S. pyogenes* strain AP1 was incubated with treated blood, and the bacterial survival rate is shown as the number of CFU at 60 min compared to the number of CFU of the IgG control, which arbitrarily was set to 100%. The absolute survival of the IgG control varied between 78 and 84% of that of the inoculum. IdeS^{C94S} was used as a negative control. *, $P < 0.05$ compared to the control by Student's *t* test.

The finding that neutralizing antibodies against IdeS are present early during infection raised the question of whether these could be of importance for the outcome of invasive streptococcal diseases. This hypothesis was tested by analyzing IdeS-neutralizing activity of acute-phase serum samples from patients with severe or nonsevere invasive streptococcal infections. However, no such correlation between the presence of neutralizing antibodies against IdeS and the severity of invasive disease could be observed, as the capabilities of neutralizing IdeS enzymatic activity were indistinguishable between the different samples (Table 4). Thus, although neutralizing antibodies are involved in the host defense towards streptococci, the presence of antibodies against IdeS appears not to directly affect the outcome of invasive disease. However, the importance of IdeS endopeptidase activity for streptococcal survival in the human host is supported by the finding that neutralizing antibodies interfere with IdeS-mediated streptococcal survival ex vivo (Fig. 4). In contrast to results from experiments addressing the neutralizing capacity of patient IgG, IdeS activity is not completely blocked by patient antibodies, which is due to the relative decrease in the ratio of total patient antibodies present in whole blood to the amount of IdeS added (240- to 400-fold). Still, neutralizing antibodies significantly affected the ability of IdeS to interfere with Fc-mediated phagocytosis and clearly promoted survival of the bacteria in whole blood (Fig. 4).

Pharyngotonsillitis and erysipelas are streptococcal diseases with a high tendency to recur. In recurrent menstruation-associated *Staphylococcus aureus* toxic shock syndrome, the subsequent development of specific IgG antibodies against toxic shock syndrome toxin 1 protects from recurrent disease (19). The fact that patients develop antibodies with neutralizing activity against IdeS raises the questions of whether these patients are protected from recurrent disease and whether the presence of

neutralizing antibodies might protect from recurrent mucocutaneous *S. pyogenes* infections rather than from invasive disease. This hypothesis is currently under investigation.

Sequence alignment of complex II IdeS variants revealed that strains of the M28 serotype express an IdeS variant with a cysteine residue in position 257 (Fig. 1) (13), while all other variants, including complex I proteins, carry a tyrosine residue in this position (Fig. 1) (13). The role of this second cysteine residue has not yet been investigated, but the recently published three-dimensional structure of IdeS (25) shows that the cysteine in position 257 is part of a flexible loop close to the catalytic site of the enzyme (25). The potential interaction of cysteine 257 and the catalytic site might explain the reported weak enzymatic activity of this variant (1, 13) and indicates that the previous characterization of complex II protein (1, 13) might not generally apply to other complex II variants. This view is supported by the fact that the complex II variants secreted by the clinical streptococcal strains exhibit enzymatic activity. However, neutralizing antibodies might be specific for one of the two IdeS variants, but only 4 of the 17 patient samples were found to contain neutralizing antibodies specific for complex II IdeS, and complex specificity correlated with the IdeS variant expressed by the infecting strain (data not shown).

The finding that an increase in the amount of antibodies does not necessarily correlate with an increased neutralizing capacity also supports the earlier notion (15, 16) that measurements of antibody titers in patient samples are not sufficient to assess the clinical and biological importance of antibodies during bacterial infection. However, the fact that seroconversion towards IdeS leads to the inactivation of enzyme activity strongly supports the view that IdeS IgG endopeptidase activity is an important streptococcal virulence determinant.

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